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Diversity of Gut Microbiota in Autism Reveals Differential Abundance of *Prevotella* and *Akkermansia Species*

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Abstract

Background: Gut-Brain-Axis provides bidirectional communicational route; imbalance of which can have pathophysiological consequences. It is a frontier in autism research, affects 85% of autistic children (NIH report). Their microbiome has few overall microbes and smaller number of health promising microbes than their neurotypical peers. We hypothesize autism gut might play a role in manifestation of autism behaviours and on treatment, can revert back to normal behaviour considerably. The aim is to better understand to what degree gut microbiota of autism subjects differs from controls and identify bacterial species present exclusively in autism.

Materials and Methods: 16s-rRNA-sequence of autism-subjects were retrieved from the American Gut Project Archive. Taxonomic assignment was inferred by similarity based methods using Quantitative Insights Into Microbial Ecology (QIIME). Species abundance was characterized and co-occurrence network was built to infer species interaction using measures of diversity. Statistical parameters were considered to validate the findings.

Result: A total of 206 (1.8%) of American Gut Project datasets onstituted of autistic samples. Various bacteria such as *Akkermansia sp*., and *Prevotella sp*., were harboured in higher abundance in autistic children with statistical significance than in controls.

Conclusion: These findings indicate connecting-link between gut-microbiome-brain-axis and autistic behaviour which can result in improved management

Keywords: autism phenome; gut microbiome; behaviour reversal; meta-analysis; 16srRNA sequencing; operational taxonomic units (OTUs)

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1.Introduction

Gut microbiome plays a vital role in the normal functioning of the gut thus contributing to the normal health and development of an individual. They include archaea, bacteria, viruses and fungi. The initial inoculums of the bacteria are received by the mother during the time of birth [1,2] During the early development, the individual gets exposed to various environmental conditions which contribute to the complexity and diversification of the microbial community leading to a unique pattern of microbiota. Gut bacteria's are commensal with our intestinal tract and depend on the host for the nutrition and space. In turn, they are a benefit to us in many ways. The commensal bacteria's are antagonist towards potential pathogens and they enhance the immunity of an individual. Metabolites produced by them are essential for a healthy living and any dysbiosis is known to influence brain thus altering one's behaviour and resulting in various disease pathogenicity like obesity, diabetes mellitus, multiple sclerosis, schizophrenia, autism etc.

Autism, which is a neurodevelopment disorder with increasing prevalence rate of 1 in 68 children in the United States (1 in 42 boys and 1 in 189 girls) [3,4]. It arises early in the developmental stage and persists throughout the life time. According to Diagnostic and Statistical Manual of Mental Disorders [5] symptoms of autism is categorized into two groups. First, includes deficits in social communication and social interaction, while, the second category is about restricted, repetitive patterns of behaviour. It is necessary to consider above-mentioned criteria while diagnosing autism subject. One can find wide range of heterogeneity in the severity of the autism symptoms which also includes cognitive ability.

Studies show many environmental and genetic risk factors to be closely associated with autism. In a study it was found out that as parental age increased, the risk is greater for the child to be born with autism [6]. There are many teratogens which are said to play role in

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manifestation of autism one of them is the valporate which is an anti-convulsant and a mood stabilizer when consumed during pregnancy increases the risk birth defects, cognitive defects and child to be born with autism [7]. Heavy metals, alcohol and chemical pesticides are also identified to be the causative agents for the defective fetal neurodevelopment. According to Simons Foundation Autism Research Initiative, there are 23 high confidence genes and 42 strong candidate genes which are associated with autism. Common characteristics of these genes are that they play a vital role during brain development. Autism is also seen to be comorbid with other psychiatric conditions ranging from Fragile X syndrome, epilepsy etc. Gut-associated problems are commonly observed in the majority of the autism subjects. They have increased gut permeability or 'leaky gut' and altered levels of cytokines and neurotrophins suggesting that they harbour varied gut microbes when compared to healthy individuals.

Earlier knowledge of microbial diversity in humans was limited as the study was purely based on the culture-dependent manner which had certain drawbacks. With the advent of modern molecular techniques, easy characterization of the microbiome without the need to culture them in the laboratory is made possible. 16s rRNA gene sequencing is the culture-independent technique which is considered to be a gold standard for microbial profiling.

A study conducted to check the abundance of *Bifidobacteria* species and the mucolytic bacterium *Akkermansia muciniphila* showed less abundance of these bacteria in children with autism [8]. One of the studies showed less diverse gut microbial composition with lower levels of *Prevotella, Coprococcus,* and unclassified *Veillonellaceae*[9]. Another study showed a significant increase in several mucosa-associated *Clostridiales*, whereas a decrease in *Dorea, Blautia and Sutterella* was seen in AUTISM-FGID [10].

The pattern of microbiome present in autism subjects vary depending on the diet, age, environment and the ethnic group. In order to understand how microbes influence autism, it is

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necessary to understand the microbial makeup of an autistic person and to have knowledge of how different is it when compared to a normal individual. This paves ways to invent novel and potential therapeutic interventions for autism. To better understand all these aspects authors made an attempt to look into the abundance of those gut bacteria in autistic subjects which are believed to possess a potential link with the pathophysiology of autism and hypothesised that the autism gut differs variably from the neurotypical peers which greatly impacts the autism phenome.

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2. Materials and Methods

The materials for the study were retrieved from American Gut Project with study accession PRJEB11419. It is the largest crowd-funded project study co-founded by Rob Knight and Jeff Leach. Samples collected from the participants are processed in Rob Knight's lab at the University of California, San Diego. Processing of the sample starts by isolating DNA from the sample which is then subjected to polymerase chain reaction to amplify 16s rRNA gene. Amplified product will be tagged with unique barcodes for each individual sample. This allows for the easy identification of which bacterial DNA belongs to which of the sample. These amplicons are then sequenced using Illumina MiSeq sequencer. The detailed workflow has been included. (Supplementary Fig1).

The sample data was taken from European Nucleotide Archive (ENA) where 11,296 samples from the American Gut Project were deposited. Samples were initially screened for autism trait, by going through attributes of every individual sample. We found 206 samples to be autistic with other comorbidities like epilepsy, Alzheimer's, seizure, IBD, schizophrenia etc. Out of which 30 classical autism samples were considered for the study based on inclusion and exclusion criteria like age, no other comorbidities, diet etc. 30 samples with normal attributes were taken from the same project as controls for the study. Demultiplexed raw sample data files were retrieved in FASTQ format from ENA i.e the primer and barcode sequences were removed and quality filtering was performed.

Various specific bioinformatics analysis pipeline such as NINJA, Lotus, MG-RAST, QIIME and Illumina inbuilt MiSeq Reporter software, QIIME, Explicet were evaluated and assessed for microbial profiling analysis. Following successful evaluation, comparative metagenomic data analysis was carried out using Quantitative Insights Into Microbial Ecology (QIIME 1.8.0) and Explicet. QIIME is an open source software pipeline able to

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perform a wide range of analysis on microbial communities sequence alignment identification of OTUs, elaboration of phylogenetic trees, and phylogenetic and taxon based analysis of measures of diversity both within (alpha diversity) and between samples (beta diversity).

2.1 Bioinformatics Analysis:

Preprocessing step:

The following parameters were set for both QIIME and MG-RAST: (i) a minimum average quality Phred score of 33 allowed in reads; (ii) a minimum and maximum sequence length in the range of 43 – 67872 nucleotides. In addition, to be as stringent as possible, we did not allow any primer mismatches (setting the parameter ―primer mismatches = 0) and allowed only a 1.5 maximum number of errors in barcodes. Removal of adapters, PCR primers and low-quality reads is essential for effective analysis. Chimeric sequences can be generated when the 16s region of interest is incomplete and the resultant partial resultant serve as primers that combine heterologous molecule with similar 3' moiety. Such chimeric sequences were detected and removed using USEARCH tool.

2.2 16s rRNA detection, clustering and identification:

The FASTQ files were converted into input files in FASTA format. The mapping file was generated which contains information for understanding what is in each sample and is required for performing the downstream analysis. It is in tab-delimited text format. The main information in this file is a unique identifier for each sample, the barcode and primer used for each sample, and a description for each sample which is necessary for understanding the results. Analysis of microbiome datasets began by a process called OTU clustering with the help of uclust algorithm, where raw sequence reads are grouped into operational taxonomic units (OTUs) based on sequence similarity threshold. Each of these clusters represents a taxonomic unit of bacterial genus or species. OTU clusters with 97% similarity threshold are

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considered at genus level while 98% or 99% identity is set for species separation. OTU clustering can be performed in three different ways: closed reference, *de novo*, and open reference. In the closed-reference approach, the sample sequences are paired and clustered against a reference sequence database. In *de novo* clustering, the sample sequences are grouped based on pairwise similarity among all sequences in the dataset. The open-reference approach is by doing a closed-reference step, which is followed by a *de novo* step. For the study, we considered closed reference based OTU clustering because the reference database for human gut microbiome is well established. UCLUST is the best-suited algorithm supported by QIIME for closed-reference OTU clustering. The QIIME script used for closed reference based OTU picking is *pick_open_reference_otus.py*

-o $PWD/open_ref_otus -i $PWD/seqs.fna -r $PWD/gg_12_10_otus/rep_set/97_otus.fasta -a -O 8

2.3 Taxonomical classification of bacterial sequences:

Comprehensive reference databases have been compiled for annotation of sequenced bacterial metagenomes which includes Green genes database, Ribosomal Database Project and SILVA. For better specificity, there are projects Human Microbiome Project which hosts a curated collection of microbial sequences associated with the human body including eukarya, bacteria, viruses, archaea from various sequencing projects. This increases the resolution of taxonomical classification of sequences. In this case, taxonomic inference for the observed OTU was assigned using Green genes reference database. Taxonomy assigned OTU list was used to build OTU table in Biological Observation Matrix (BIOM) format, which is required for downstream analysis. Summarising the taxa was performed using the script *summarize_taxa.py –i otu_table.biom –o taxa_summary/ -m mapping_file*. This is for visualizing the different bacteria present in samples and their relative abundances.

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2.4 Diversity analysis:

Overall diversity analysis for subsequent comparative and statistical evaluation was obtained. Measures of diversity- alpha diversity (within sample diversity) and beta diversity (between sample diversity) were calculated. It was ensured that rarefaction of the sample was performed to maintain even depth reads to avoid false positive outcomes. Minimum rarefaction was chosen depending on the minimum number of sequence per sample. Using various metrics like PD_whole_tree, chao1, observed_otus, observed_species alpha diversity was computed for each rarefied OTU Table. Beta Diversity is a method to study similarities and dissimilarities between two datasets. There are two main approaches for quantifying β-diversity: phylogenetic β-diversity [11] and non-phylogenetic methods (Kuczynski et al., 2010). With phylogenetic methods, differences in abundances that involve closely related species are given lower weights, on the assumption that closely related species have similar genetic capabilities. One example is UniFrac (unique fraction), which has been reported to correlate well with the biological properties of samples [12] and measures the amount of unique evolution of a community in comparison to others. Phylogenetic metrics are reliant on the quality of the constructed tree for the bacterial communities within the samples, which can be problematic in some cases, contingent on the taxa and the 16S rRNA gene variable region used. One of the most popular non-phylogenetic approaches to quantify β-diversity is the Bray-Curtis dissimilarity [13, 14]. Finally, the obtained distance matric was used to compute Principal Coordinate Analysis and converted into plots for result visualization using Emperor.

2.5 Statistical Analysis

Various statistical analysis parameters like P-value, Standard Deviation, and Standard Error were calculated to ensure that the results obtained are statistically significant.

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3. Results

Out of 11,296 samples screened from the American Gut Project, we found 206 samples to be of autism. Some of these samples contained other comorbidities like ADHD, autoimmune, *Clostridium difficile*, schizophrenia, diabetes mellitus, epilepsy, IBD, IBS etc. This constitutes ~1.8% of American gut project participants to be autistic.

Samples were subjected to OTU clustering step which produced 4372 OTUs in autism samples while 4370 OTUs in control samples. It provided a detailed account about the OTUs present in each of the sample. These OTUs were assigned taxonomy considering the reference as Greengenes dataset with 97% similarity. Percentage of various bacteria resides in the autistic gut and may affect the host health adversely. OTU Table with all the OTUs present in the sample was used to build the BIOM Table which was used to summarize the taxonomy. This helped in visualizing the OTUs present in the sample. Taxa summary for the relative abundance of taxa in the sample using different taxonomic levels like phylum, class, order, family, genus and species was performed. This provided an idea about the similarities and dissimilarities among sample sets. Output files also contain bar charts and area chart with their legends and relative abundance.

Firmicutes, Bacteriodetes, Tenericutes and Fusobacteria were higher in abundance in autism samples by 7%, 3.5%, 1% and 0.7% respectively. While Proteobacteria, Actinobacteria and Verrucomicrobia were higher in abundance in control samples by 10.8%, 0.7% and 0.6% respectively. Euryarchaeota, Cyanobacteria and Synergistetes are showing the difference by 0.1%.

3.1 Taxa summary

The gradient of varied abundance at the phylum level was observed across the case and control samples. Phylum Firmicutes was harboured at a significantly higher abundance in cases (45.6%) than control samples (38.6%). In contrast, the Phylum

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Proteobacteria was in higher abundance in control (31.4%) than that of cases (20.6%). Synergistetes was present only in control samples, while Euryarchaeota, Cyanobacteria, Fusobacteria are exclusively present in autism samples with 0.1%, 0.1% and 0.7% abundance respectively.

Even though we see a particular phylum to be present in all the control and case samples when we look into species belonging to the same phylum we see different species abundance across cases and control samples. For ex: In accordance with Firmicutes trend in Phylum, significantly higher abundance is harboured by *Streptococcus* belonging to Firmicutes in cases. Genus *Bacteroides* is found to be present in all the samples (Fig 1).

Fig 2 gives an idea of the diversity within the data sets. On the X coordinate, we find sample size while on the Y co-ordinate various matrices like Chao1, ShannonH, Goods, and Simpson. Few of the case and control samples are grouped together signifying the similarity between them. While some of the case and control sample are isolated and fall into different coordinates, suggesting that these samples are different from the rest of them. Manhattan plot displays logarithmically transformed p values with higher peaks representing lower p values. The horizontal lines represent p values of 0.10, 0.05 and 0.01 inclusion of the p=0.10 is intended to highlight taxa that are approaching significance in the analysis. In the Manhattan plot, the first significant peak (position 380) corresponds to *Prevotella* which have higher proportions and relative abundance in the cases. (Fig 3)

A mixture of red and black could be seen on the heat map suggesting that the species abundance is similar across controls than across cases vs controls. Anatomical positions with Morisita Horn values are near to 1 implying the samples constituent taxonomy patterns are similar and red indicates differential taxonomy in the samples (Fig 4).

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Taxa summary gave ~230 genera present in case and control samples, out of which, five were selected for the study considering their relative abundance and their relatedness to autism. *Prevotella sp.* and *Veillonella sp.* were in higher abundance in autism samples by 4.9% and 2.7% respectively. *Akkermansia sp.* was in lower abundance by 0.6% in autism sample. *Ruminococcus sp.* and *Sutterella sp.* were evenly distributed among the case and control sample (Table 1).

The generated heat map for OTUs gave a clear picture that most of the species abundance in the samples are conserved, depicted in dark green coloration. Interestingly, a couple of blocks were observed and identified which were unique to few samples and common across the rest.

3.2 Gut Metabolite Pathway:

Interaction network pathway was built using bacterial metabolites and gene targets and associated pathways involved in human metabolism with the help of Ingenuity pathway analysis (Fig 5).

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4. Discussion:

In the aim of the study was to better understanding of the differential bacterial species abundance across cases and control, the authors identified that 1.8% of the population contains autistic subjects with GI problems. The subjects taken for the current study showed the presence of certain bacterial species in abundance while some species were exclusively present in autistic subjects. This suggests that the autistic children harbour elevated bacterial populations which could be the reason behind the anxiety, behavioural differences and other irritational consequences. A research group at the University of California have performed various mice model based studies wherein the knockout mice with autistic behaviour was maintained and reversal of autistic behaviour was observed on treatment of mice with the *Bacteroides fragilis*. This supports the directionality that bacterial interventions can be a boon to autism research and act as a therapeutic approach to better manage the disease.

Taxonomic classification depicted the prevalence of a wide variety of bacteria, archaea in the samples. Various statistical approaches provided confidence to the data and measures of diversity and other parameters helped in depicting species abundance within and between samples which hold importance for better lineage and characterization of the bacterial population.

Pathway enrichment would help in connecting dots from bacteria to metabolites present in gene mutations and give a holistic picture and approach wherein bacterial species and metabolites can be traced back to the genomic variants and study the etiology of the disease in an elusive manner. Differences reported on microbial diversity and composition can also be attributed to the fact that several microorganisms can perform the same function.

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This metabolic function can be assessed by measuring metabolites produced. Several published metabolomics analyses of urine and fecal metabolites have revealed a differential abundance of bacteria-produced metabolites that have the potential to directly affect neural processes. In the present study, a pathway involved in various metabolites from bacteria was constructed and mapped to various pathways and gene mutations in the affected individual. Succinic acid is the metabolite produced by the gut bacteria *Prevotella sp.*, succinic acid is known to degrade Glutamate. Glutamate which is a neurotransmitter plays a role in learning, memory, they also act to influence earlier developmental events, some of which occur prior to synapse formation: such as proliferation, migration, differentiation or survival processes during neural development. (Lujan et al., 2005)

Akkermansia sp was found to be in lesser abundance in autism samples. This bacteria is known to produce mucosin which is necessary to maintain the mucosal layer in the gut lining. *Prevotella sp* was found to be in higher abundance in autism samples. Studies suggest that the metabolism of protopanaxadiol saponins to metabolites I—III in the intestines seems most partly due to intestinal *Prevotella sp.* (Hasegawa et al., 1997). The coexistence of protopanaxadiol (PPD) and protopanaxatriol (PPT) type ginsenosides in ginseng may be associated with its dual effects that can both stimulate and sedate the CNS (Wee et al., 2011).

Emerging studies suggest the microbiota is an important regulator of GI physiology, immune function, and behaviour. Abnormalities in each of these domains are reported in autism, but the additional characterization of comorbid medical symptoms is required to clarify the nature, strength, and reproducibility of specific associations. Evaluation of genetic background, medical history, and autism severity, among other variables, would provide insight into whether particular symptoms are enriched in specific

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subtypes of autism and would further drive hypotheses regarding possible contributions of microbial dysbiosis, GI dysfunction, or immune dysregulation to the development or persistence of autism behaviours. Similar efforts to characterise comorbid microbiota, GI and immune symptoms across new and existing animal models for autism are needed, with an emphasis on identifying converging phenotypic signatures across models of different genetic and environmental autism risk factors. Further experiments are required to determine whether microbiome, GI, or immune abnormalities can sufficiently cause primary behavioural features of autism. Such investigations should begin with studies using gnotobiotic or xenobiotic animals to identify peripheral targets and specific brain changes for the development of novel autism therapeutics. Of particular relevance to autism-related microbial dysbiosis studies, it would be important to determine whether fecal transplant of autism microbiota into animals is sufficient to cause behavioural impairments, neuropathologies, and medical comorbidities seen in autism. Moreover, well-controlled studies on the efficacy of fecal microbiota transplant in autism patients would provide much-needed guidance to the autism community.

Conflict of Interests- None

The authors declare no competing financial interests.

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Ethics Statement

Written consent was obtained from all participants involved in this study and the Institutional Human Ethical Committee (IHEC UOM No.128 PhD/2016–17) approved the consent procedure.

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Figure legends

- Fig 1: Gut microbial diversity of autism cases and control at (a) phylum and (b) species level
- Fig 2: Alpha diversity graph for case and control samples using NINJA-OPS and QIIME respectively
- Fig 3: Manhattan plot depicting peaks which corresponds to species abundance in various samples using a.) QIIME data and b.)NINJA-OPS data
- Fig 4: Heatmap of beta diversity of gut microbiota a) Overall and b) Prevotella in autism cases and controls reveals blocks of different gradient of divergence
- Fig 5: Pathway showing the interaction between the gut bacteria and their metabolites

Table Legend

Table 1: Relative abundance of enriched bacterial genera in the fecal microbiota of autism cases and control using QIIME pipeline